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Human mucin glycoprotein MUC1 is normally expressed on the apical surface of ductal epithelial cells. MUC1 is overexpressed all surface of a wide variety of ductal adenocarcinomas, including those of breast, pancreas, lung, colon, and prostate [1]. Thus MUC1 is a potentially attractive immunogen for a cancer vaccine with broad specificity. MUC1 is essentially "self" antigen, and, as such is only weakly immunogenic. Natural responses against MUC1 are characterized by a low frequency of cytolytic T lymphocytes (CTL) and low titers of antibodies. We hoped to enhance the immune response to MUC1 antigen by using mimics of natural MUC1 antigen. Mimics are peptides of a different structure from the natural peptide, but which can stimulate or re-stimulate a T cell response to the latter. Such responses may be an augmentation or a diminution, depending upon the mimic. The activity of mimics takes advantage of the degeneracy of T cell recognition, i.e. on the fact that a single T cell receptor (TCR) can recognize many different peptides.

To develop MUC1 specific mimic peptides we planned 1) to establish a MUC1 specific CTL, 2) to use combinatorial peptide libraries in a positional scanning format to determine which amino acid substitutions in the original peptide were recognized best by CTL [2], 3) to synthesize those mimics; test them on index cell line and identify those that were stronger immunogens for CTL than the native peptide.

First we had to choose the best (most immunogenic) native MUC1 peptide and develop T cell line from a healthy HLA-A*0201+ (HLA-A2) individual. Such a line had to be (1) highly specific for chosen peptide, 2) strongly cytotoxic against MUC1 positive adenocarcinoma cells, and (3) contain at least $10^8$ T cells. All of these qualities are required for successful analysis of the peptide library. Because MUC1 is a weak "self"

antigen, this first step proved to be exceedingly difficult. We tested a number of HLA-A*0201 restricted MUC1 epitopes described in the literature, such as SAPDTRPAP, APDTRPAPG, STAPPAHG (Compagno, D. and Mitchell, M.S., unpublished data) or 3) STAPPVHNV [3] with or without PADRE T helper peptide as an adjuvant, but were unable to develop a stable CTL line. Resulting lines were either non- cytotoxic, or grew poorly with few cells. More then 20 CTL lines were established but none met our criteria. Finally however we were successful in developing a stable T cell line against a peptide in the leader sequence of MUC1 (amino acids 12-20, LLLLTVLTV), previously described by Brossart et al. [3]. This CD8+ CTL line (named CCM4) was characterized by strong specific cytotoxicity against T2 cells labeled with specific peptide (Figure1), as well as good sensitivity. T cells could recognize 10-50 ng/ml of peptide (Figure 2; ELISA, similar data on $^{51}Cr$ release, not shown). CCM4 was both cytotoxic and secreted a large amount of IFN-γ, which allowed us to use it in $^{51}Cr$ release and IFN-γ secretion assays. In response to specific stimulation almost 100% of CCM4 T cell synthesized IFN-γ, which demonstrated that the line consists exclusively of MUC1 peptide-specific cells (Figure 2, surface IFN-γ expression). This feature is important for scanning of the positional library, because non-specific ballast cells within the T cell line may produce non-specific "noise" that would make it difficult to interpret the data. CCM4 demonstrated strong, MHC class I restricted cytotoxicity against the HLA-A2+ breast cancer line MCF7 (Figure 3). T cells were strongly cytotoxic against HLA-A2 matched MCF7, but not against the HLA unmatched M3 melanoma cells; and cytotoxicity could be blocked by pre-incubation with anti HLA class 1 W6/32 antibodies. Hence, killing was HLA class I restricted. More importantly, lysis HLA-matched tumor cells showed

that the peptide we chose for the *in vitro* immunization and determination of mimics was relevant to anti-cancer immunity. Of importance, CCM4 grew well after stimulation.. Thus far approximately 2 X $10^8$ T cells were produced with about 5 X $10^7$ frozen for future studies, and the line is still thriving in culture. This feature is very important, since a low number of T cells in specific lines is one of the major obstacles in the use of positional scanning libraries, the strength of whose signal depends upon the release of $^{51}Cr$ or IFN-$\gamma$.

With the CCM4 cell line we were able to screen successfully a combinatorial peptide library. This procedure was repeated 3 times, for some crucial positions 4 times. Results of typical scanning are presented in Figure 4. It is evident from this scan that index line successfully recognized native amino acids in their correct (native) positions (p1-L, p4-L, p.9 –V etc.) It is also evident that native amino acids can be substituted with possible enhancement of immune response (p.1 L⇒V, pos.2 L⇒I, pos.3 L⇒N etc). As a result of scanning, amino acid substitutes that did not impair recognition of the peptide by CTL were also determined. 130 mimic peptides have been synthesized. At the moment we are in process of analyzing the potency of those mimics on stimulating the index CCM4 line. Then we will use the mimics to immunize naïve HLA-A2+ lymphocytes to determine mimics with stronger agonist activity than the native peptide. As a necessary preliminary investigation, while we were developing the appropriate CTL line against MUC1, we analyzed the 9-mer tyrosine melanoma peptide YMNGTMSQV (Figure 5). After similar library analysis, synthesis of candidate mimic peptide and test on an index CTL line we were able to identify several agonists of the original tyrosinase peptide that were more immunogenic. The strength of immunogenicity was expressed in either of two

ways: some mimics stimulated CTL in lower concentration than the native peptide, while others caused much stronger response than the native peptide at similar concentrations. Such mimics are good candidates for clinical immunization against melanoma. We plan to identify similar mimics to MUC1 peptide in the immediate future.

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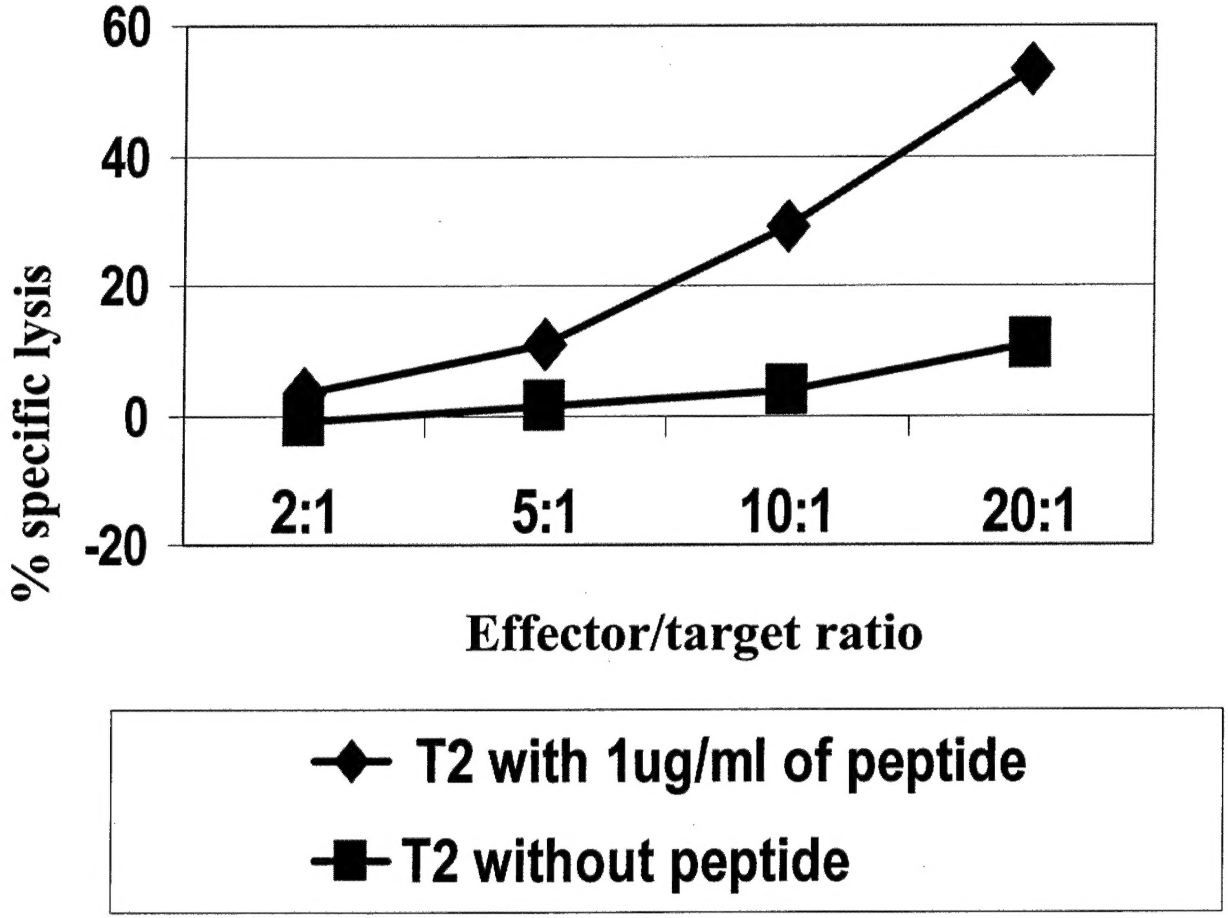
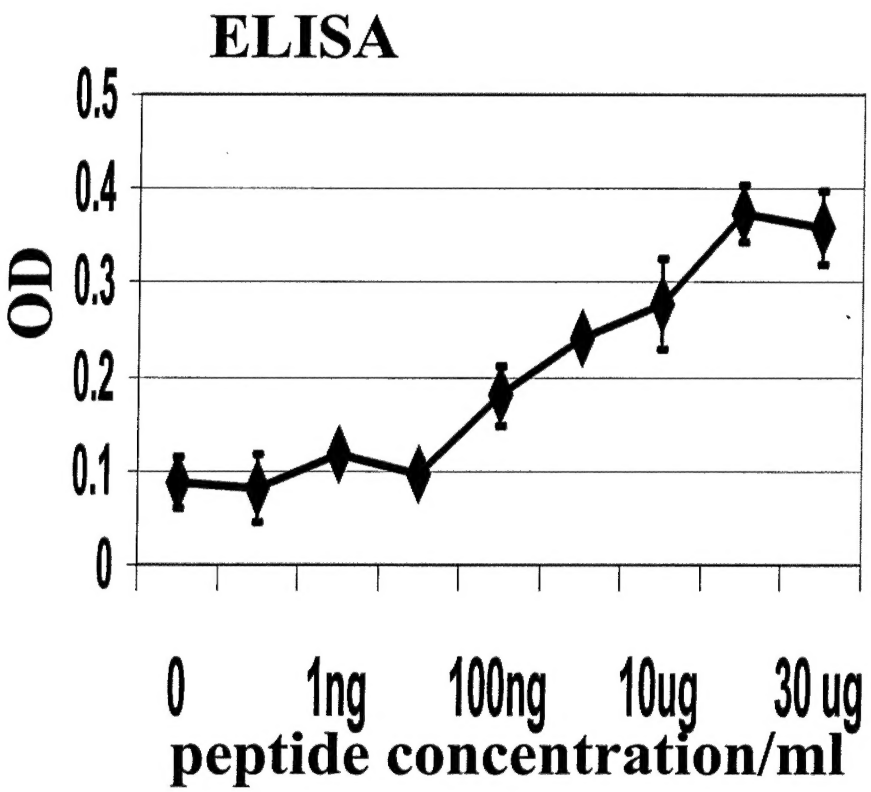
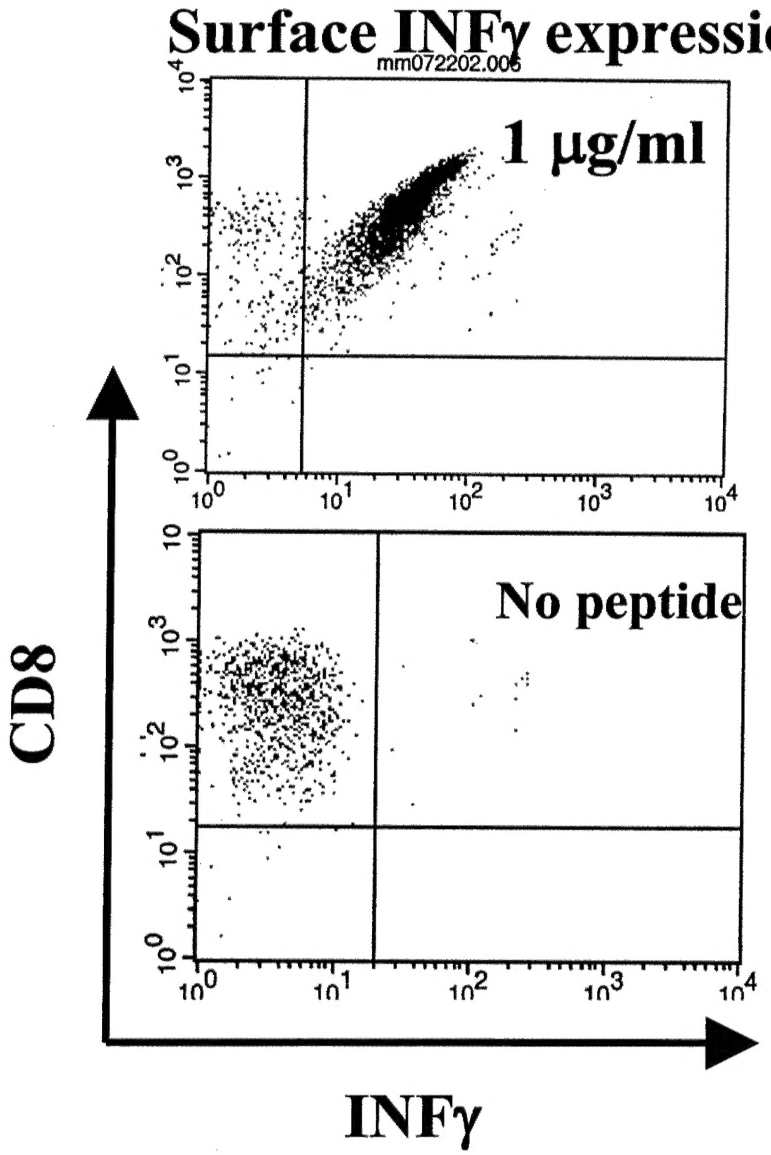
# *CCM4: CTL line against MUC1* $_{12\text{-}20}$ *signal sequence* **LLLLTVLTV**

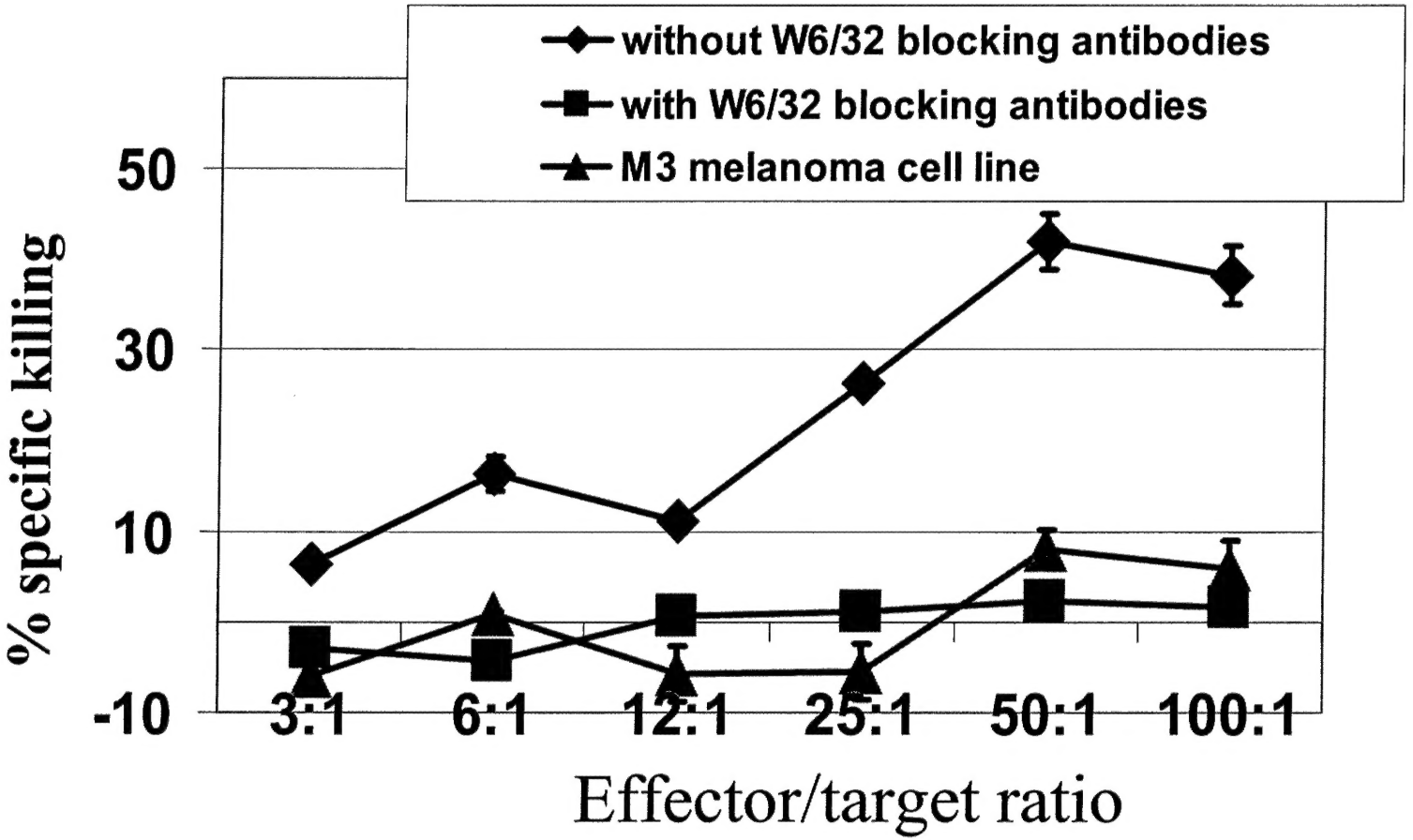


# CCM4: *INFγ Secretion with Specific Stimulation*

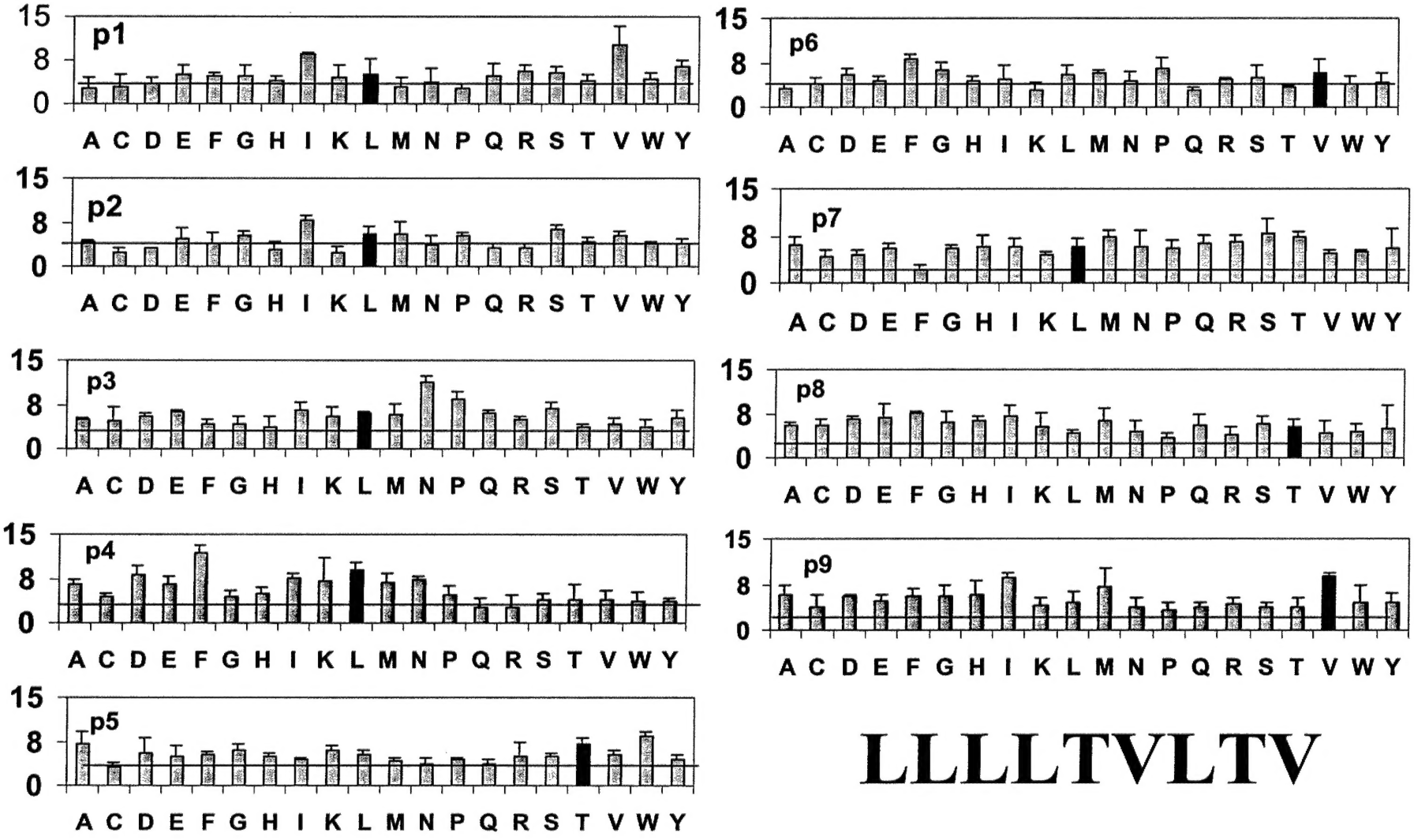


# **CCM4:** *MHC class I restricted cytotoxicity against MCF7 breast cancer cell line*



# *Positional scanning synthetic combinatorial nonameric library*




**LLLLTVLTV**

# Predicted peptides can be better recognized by index CTL than original one